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Effect of different sensitizing doses of antigen in a murine model of atopic asthma

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SUMMARY

The dose of antigen is assumed to be one of the important factors in the polarized development of helper T cell subsets, i.e. Th1 or Th2 cells. We investigated the effect of the sensitizing antigen dose in a murine model of atopic asthma, which involved sensitization with ovalbumin (OVA) followed by repeated exposure to OVA aerosols. BALB/c mice were primed with varying doses of OVA (0, 10, 100 and 1000 μg) plus $Al(OH)_3$ on days 0, 7 and 14, and were challenged with OVA aerosols (50 mg/ml for 20 min) on days 15–20. There were striking antigen dose-related differences in OVA-specific antibodies: high IgE and low IgG2a titres were found in mice sensitized at 10 μg, while low IgE and high IgG2a titres were seen at 1000 μg. The sensitizing dose was inversely correlated with the total cell count and the eosinophil count in bronchoalveolar lavage fluid (BALF), as well as with the extent of histological changes such as goblet cell hyperplasia of the bronchial epithelium and cellular infiltration into bronchovascular bundles. Antigen-induced bronchial hyper-responsiveness (BHR) to methacholine was observed with sensitization at 10 μg but not at 1000 μg. Splenic mononuclear cells (SMNC) obtained from mice sensitized at either dose showed proliferation in response to OVA. Production of IL-4 and IL-5 by OVA-stimulated SMNC was inversely correlated with the dose of sensitizing antigen. High-dose sensitization resulted in general suppression of cytokine production by SMNC, including interferon-gamma (IFN-γ). The BALF levels of IL-4 and IL-5 were increased by low-dose sensitization, whereas IFN-γ and IL-12 levels were increased by high-dose sensitization. These results suggest that the dose of sensitizing antigen defines the phenotypic changes in the present murine asthma model, presumably by influencing the pattern of cytokine production.

Keywords cytokine eosinophilic inflammation Th1 Th2 bronchial hyper-responsiveness

INTRODUCTION

Murine $CD4^+$ T cell clones can be divided into two functionally distinct subsets, termed Th1 and Th2 [1,2]. These subsets are distinguished by the different cytokines that they produce. Th1 cells characteristically secrete IL-2 and interferon-gamma (IFN-γ), and are involved in cell-mediated immunity, whereas Th2 cells secrete IL-4, IL-5 and IL-10, and are involved in immediate allergic reactions and humoral immunity. This functional dichotomy of $CD4^+$ T cells has been demonstrated to exist in humans [3]. Dominance of one or the other effector cell type has been implicated in a variety of diseases, including bronchial asthma. The pathophysiology of atopic asthma involves various inflammatory cells and cytokines [4,5], and it is characterized by eosinophilic bronchitis, an elevated serum IgE level, and bronchial hyperresponsiveness (BHR). Among Th2 cytokines, IL-5 is an essential factor for eosinophilic inflammation [6–8], and IL-4 has a pivotal role in IgE production [9,10]. In contrast, IFN-γ (a Th1 cytokine) is known to inhibit both eosinophil recruitment and IgE synthesis [11,12]. Therefore, atopic asthma could be considered as a Th2 cell disease, in which specific allergens induce a Th2-type response in genetically predisposed (atopic) people. In fact, Th2-like cells have been demonstrated in the lungs of atopic asthma patients [13,14].

Elucidation of the precise mechanisms involved in the induction of Th1 or Th2 dominance is important for understanding the pathophysiology of asthma, and for developing efficient methods of treatment and prevention of the disease. The Th1/Th2 balance has been reported to be influenced by several factors, such as the genetic background, the adjuvant employed, the accessory cells, and the cytokines present during early T cell activation [15–21]. IFN-γ can direct the differentiation of Th1 cells. IL-12 can also influence the development of Th1 cells, and the level of IL-12 receptor expression differs between Th1 and Th2 cells. On the other hand, the presence of IL-4 or IL-6 favours Th2 differentiation. It has also been reported that the dose of antigen influences the

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antibody response, which may be relevant to the polarized development of Th1 and Th2 cells [22–24]. More recently, Hosken *et al*. observed that midrange doses of peptide antigens induced the development of Th1-like T cells in a study using naive $CD4^+$ T cells from T cell receptor (TCR) $\alpha\beta$-transgenic mice ($OVA_{323-339} + IA^d$; BALB/c) [25], while very high and very low doses of the peptides caused a dramatic switch to the development of Th2-like T cells. Conversely, Constant *et al*. demonstrated that higher antigen doses favoured Th1 development, whereas lower doses induced Th2 development in naive $CD4^+$ T cells from another type of TCR $\alpha\beta$-transgenic mouse (pigeon cytochrome c + H-2^b; B10.A(5R)) [26]. These *in vitro* observations clearly indicate that the antigen dose is one of the determinants of the polarized development of Th1 and Th2 cells.

Recently, several murine models of atopic asthma have been described [27–29]. In these models, multiple sensitization and repeated transbronchial challenge are the standard procedures to induce a Th2-dominant response, such as a highly specific IgE response and pulmonary eosinophilic inflammation. There have been many studies using such murine models to investigate the role of $CD4^+$ T cells, mast cells, costimulatory molecules, and mediators such as thromboxanes and leukotrienes [4,29–32]. The common protocol for such models includes sensitization with a low dose of ovalbumin (OVA). We used such a murine asthma model to study the *in vivo* influence of the sensitizing dose of antigen on the production of specific antibodies, as well as histological changes such as eosinophilic infiltration and epithelial damage, the BHR to methacholine (MCh), and the profile of cytokine production.

MATERIALS AND METHODS

Mice and experimental protocol

Sensitization and challenge were performed as described previously, with some modifications [29]. Mice (BALB/c, 6–8-week-old females; Charles River Labs, Kanagawa, Japan) were primed intraperitoneally with different doses (0, 10, 100 or 1000 μg) of OVA (Grade V; Sigma Chemical Co., St Louis, MO) and 40 mg $Al(OH)_3$ in 0·2 ml PBS pH 7·4. Injections were given three times on days 0, 7 and 14. The primed mice were individually placed in a 50-ml plastic tube and challenged by repeated exposure to an aerosol of OVA (50 mg/ml), which was delivered by a DeVilbiss 646 nebulizer (DeVilbiss Corp., Somerset, PA) driven by compressed air at 5 *l*/min. Challenge was done for 20 min once a day for 6 consecutive days (days 15–20).

Mice were killed by cervical dislocation under anaesthesia at 24 h after the last challenge (day 21). Bronchoalveolar lavage fluid (BALF; 0·8 ml × 6) was obtained from the whole lungs by cannulating the surgically exposed trachea for cell analysis. In a different set of mice, a small volume of BALF was obtained (0·5 ml × 3) and stored at −80°C until analysis. Cells in the BALF were counted using a haemocytometer. The differential cell count was obtained using cytospin preparations (May–Giemsa staining). For histological examination, the lungs were removed and fixed in 4% paraformaldehyde. Then the tissues were embedded in resin (Jung HistoResin Plus; Reichert/Jung, Heidelberg, Germany) and cut into 2-μm sections, which were stained with haematoxylin and eosin (H–E), or periodic acid-Schiff.

Measurement of BHR

The BHR of freely moving mice to aerosolized MCh was measured just before BAL by whole-body plethysmography (Buxco, Troy, NY), as described previously [33]. Before taking readings, the box was calibrated by rapid injection of 100 ml air into the main chamber. Pressure differences between the main chamber containing the mouse and a reference chamber were recorded. Readings were obtained at baseline and after exposure to aerosolized saline or MCh (3–25 mg/ml). Data were averaged for 3 min, and expressed as the 'enhanced pause' (Penh): $Penh = [(Te - Tr)/Tr] \times (PEP/PIP)$, where Te is the expiratory time (s), Tr is the relaxation time (time of the pressure decay to 36% of total box pressure during expiration), PEP is the peak expiratory pressure (ml/s), and PIP is the peak inspiratory pressure (ml/s). Results are expressed as percentage increase of Penh following challenge with each concentration of MCh, where baseline Penh (after saline challenge) was expressed as 100%.

Determination of specific antibody levels

OVA-specific IgE and IgG2a antibody titres were determined by indirect ELISA [28]. Plates (Costar, Cambridge, MA) were coated with 200 μg/ml of OVA diluted in 0·1 M $NaHCO_3$ (pH 8·3). Following overnight incubation at 4°C, the plates were washed three times and blocked with 1% bovine serum albumin (BSA; Sigma) in PBS for 2 h at 37°C. Serum samples were serially diluted in 1% BSA–PBS, and were applied to the blocked wells and incubated overnight at 4°C. Then the plates were washed and were incubated for 2 h at 37°C in horseradish peroxidase-conjugated rat anti-mouse IgE and IgG2a MoAbs (PharMingen, San Diego, CA) diluted in 50% goat serum (GIBCO, Grand Island, NY) in PBS. After washing, wells were developed using OPDA solution (0·3% *o*-phenylenediamine dihydrochloride, 0·02% H_2O_2, 0·15 M citrate buffer, pH 4·9) and the absorbance was determined at 492–620 nm using a microplate autoreader. As an internal standard, pooled serum from OVA-immunized BALB/c mice was included in each assay. The standard serum was assigned a value of 100 U/ml for both OVA-specific IgE and OVA-specific IgG2a.

Assessment of splenic mononuclear cell proliferation and measurement of cytokines

The spleens of mice were removed aseptically and splenic mononuclear cells (SMNC) were purified by a density-gradient method using Lympholyte-M (Cedarlane Labs, Ontario, Canada). Then the cells were suspended in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS; GIBCO), 100 U/ml penicillin G, 100 μg/ml streptomycin, 50 mmol/*l* 2-mercaptoethanol (2-ME; Sigma), and 10 mmol/*l* HEPES (Sigma). Cells were seeded ($2 \cdot 5 \times 10^6$/ml) in 96-well flat-bottomed plates for the proliferation assay and in 48-well plates for collecting supernatant. For stimulation of the cells, OVA (10, 100 or 1000 μg/ml) was added to the cultures. SMNC were cultured for 72 h at 37°C in a humidified incubator with a 5% CO_2 atmosphere. ^{3}H-thymidine (0·5 μCi/well) was added to the wells before harvesting, and its incorporation was measured by a liquid scintillation counter [34]. The supernatant was collected at 48 h and stored at −80°C until assay.

IL-4, IL-5, IL-12, and IFN-γ ELISA kits were purchased from Endogen (Boston, MA). The concentration of each cytokine in the SMNC supernatant or BALF was measured according to the procedures recommended by the manufacturer. The detection limit was 5 pg/ml for IL-4, IL-5, and IL-12, while it was 15 pg/ml for IFN-γ.

Table 1. Effect of different sensitizing doses on bronchoalveolar lavage fluid (BALF) cells†

Sensitizing dose of antigen (μg/body)‡	Total cell count ($\times 10^5$/ml)	Differential (%)		
		Eosinophils	Macrophages	Lymphocytes
0	2·9 ± 0·1**	0**	99·3 ± 0·5**	0·6 ± 0·4**
10	12·9 ± 4·5*	78·0 ± 9·9*	15·6 ± 10·4*	6·0 ± 3·2*
100	9·1 ± 1·9*	66·4 ± 12·5*	25·6 ± 11·8*	7·8 ± 2·2*
1000	4·5 ± 0·8***	12·4 ± 3·0***	85·3 ± 3·0***	2·1 ± 0·7**

† BAL was performed as described in Materials and Methods and results are expressed as the mean ± s.d.
‡ $n = 6$.
Significantly different from the 0 μg group* and 10 μg group**.

Statistical analysis

Data are expressed as the mean ± s.d., unless otherwise stated. Variables were analysed using the Kruskal–Wallis test, while the Mann–Whitney *U*-test was used for comparison of two variables. Spearman's rank sum test was used for determining correlations. $P < 0{\cdot}05$ was accepted as statistically significant.

RESULTS

As shown in Table 1, sensitized mice (10, 100 or 1000 μg of OVA) had a significantly higher number of BALF cells compared with unsensitized mice (0 μg of OVA). Most of the cells were eosinophils, but lymphocytes were also significantly increased. Among sensitized mice, the 100 μg group had significantly fewer cells than the 10 μg group. Mice from the 1000 μg group showed far less cellularity than either the 10 μg or the 100 μg group.

Histological examination revealed epithelial cell damage with goblet cell hyperplasia, and there was also cellular infiltration, particularly in the bronchovascular bundles. Eosinophils and

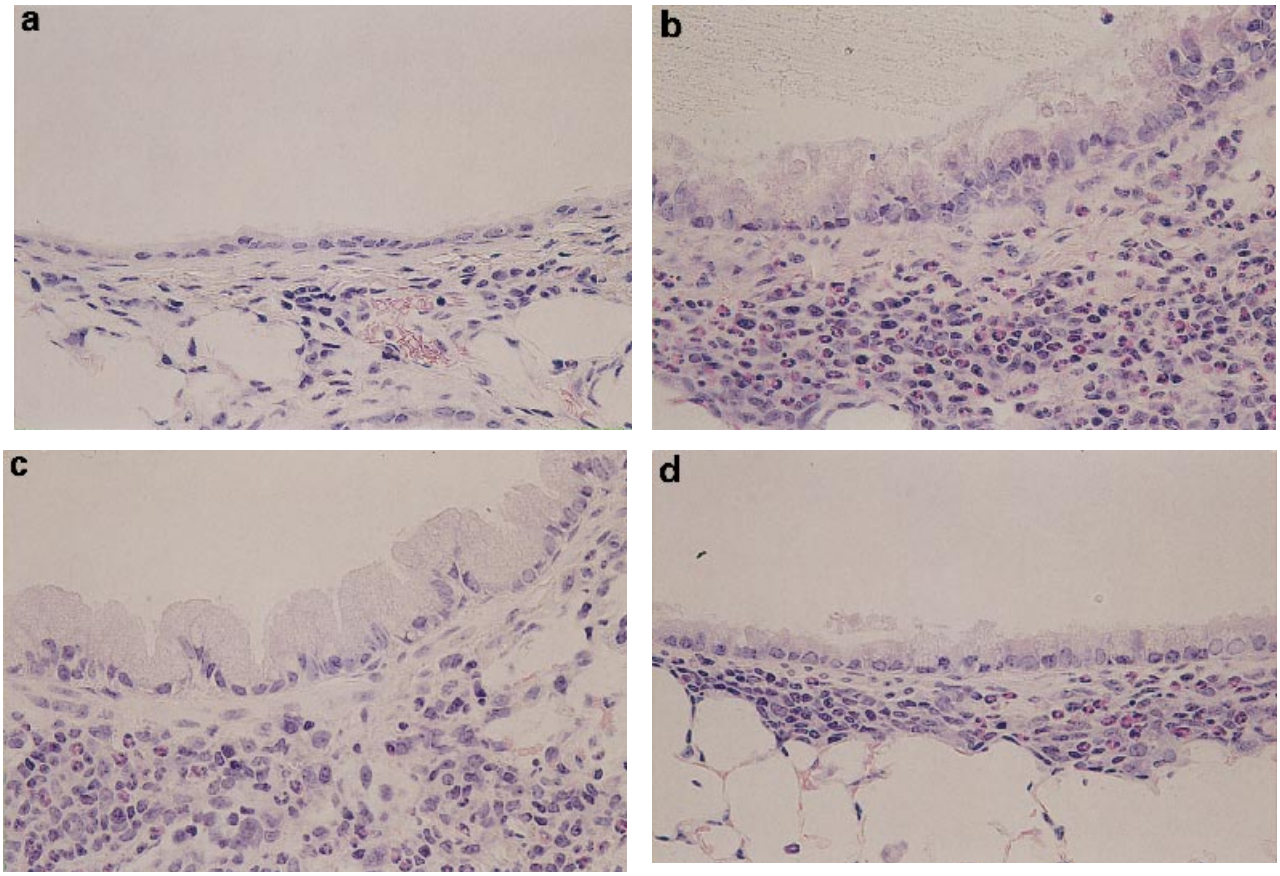

Fig. 1. Representative photomicrographs of lung specimens from each group. The sensitizing dose of ovalbumin (OVA) was: 0 μg (a), 10 μg (b), 100 μg (c), and 1000 μg (d). Marked thickening of the airway epithelium, goblet cell hyperplasia, and submucosal cell infiltration are seen with sensitization at 10 μg (b). These changes are less evident as the dose of antigen is increased. Lung sections were stained with haematoxylin and eosin. Original mag. ×400.

lymphocytes were the predominant infiltrating cells. These findings were most prominent in the 10 μg group and were least prominent in the 1000 μg group. There was no evidence of alveolitis, even in the 1000 μg group. Representative micrographs are shown in Fig. 1.

Figure 2 shows the titres of OVA-specific antibodies. In the 10 μg and 100 μg groups, there was a high titre of IgE but a low titre of IgG2a. In contrast, the 1000 μg group showed the highest IgG2a titre and the lowest IgE titre.

Figure 3 shows the results of BHR measurement. Antigen-induced BHR was observed in the 10 μg group. However, the development of BHR was almost completely inhibited in the 1000 μg group, and it was similar to that in unsensitized mice.

To investigate whether T cells from treated mice could respond to the sensitizing antigen (i.e. show a lack of tolerance), proliferation of SMNC was measured (Fig. 4). SMNC from all groups of sensitized mice showed a dose-dependent proliferative response to OVA, although it was weak in the 1000 μg group and was strongest in the 10 μg group.

As shown in Figure 5, IL-5 was detected in the culture supernatant of OVA (1000 μg/ml)-stimulated SMNC from all sensitized groups, but not in the unsensitized (0 μg) group. However, a higher dose of antigen induced less secretion of IL-5. A similar pattern was observed for the production of IL-4. On the other hand, IFN-γ was detected in all groups, including the 0 μg group. Secretion of IFN-γ was highest in the 100 μg group, followed by the 10 μg group and then the 1000 μg group. IL-12 was barely detected in the supernatants. Essentially the same results were obtained by assay of the culture supernatants from cells stimulated with different doses of OVA (100 or 10 μg/ml) (data not shown). There were significant differences in the ratio of IL-5 to IFN-γ production by OVA (1000 μg/ml)-stimulated SMNC among the groups ($1{\cdot}68 \pm 1{\cdot}11$, $0{\cdot}68 \pm 0{\cdot}31$ and $0{\cdot}25 \pm 0{\cdot}08$ for 10, 100 and 1000 μg groups, respectively; $P < 0{\cdot}05$). The BALF eosinophil count was significantly correlated with this ratio ($r_s = 0{\cdot}73$, $P < 0{\cdot}005$).

To evaluate the effect of the sensitizing dose on local cytokine production, the levels of IL-4, IL-5, IFN-γ and IL-12 were measured in BALF from mice of the 10 and 1000 μg groups (Fig. 6). IL-4 and IL-5 were detected in the 10 μg group, but not in the 1000 μg group, whereas IFN-γ was detected in the 1000 μg group and not in the 10 μg group. IL-12 was detected in both groups, but the level was higher in the 1000 μg group.

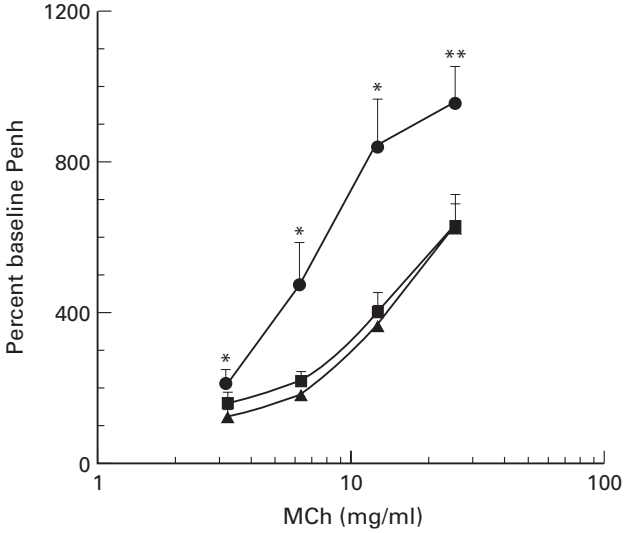

Fig. 3. Development of antigen-induced bronchial hyper-reactivity to methacholine (MCh). Results are expressed as mean percentage increase of enhanced pause (Penh) ± s.e.m. of 10 mice in each group (see Materials and Methods) following challenge with each concentration of MCh. Baseline Penh (after saline challenge) was expressed as 100%. Antigen-induced development of bronchial hyper-responsiveness (BHR) was observed in mice from the 10 μg group (l). However, in mice from the 1000 μg group (n), BHR was almost completely inhibited and was much the same as in unsensitized mice (s; PBS alone). *$P < 0{\cdot}001$; **$P < 0{\cdot}05$, compared with unsensitized mice.

DISCUSSION

Our murine model employing OVA sensitization at 10 μg displayed pulmonary eosinophilia, bronchial epithelial damage,

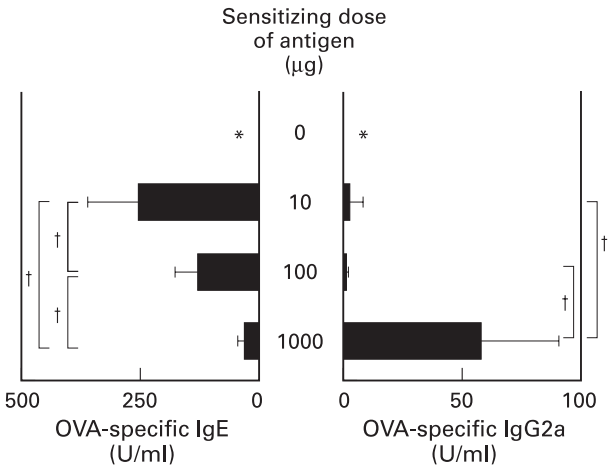

Fig. 2. Ovalbumin (OVA)-specific serum IgE titre (left) and IgG2a titre (right). Values represent the mean ± s.d. of six mice in each group. In the 10 μg and 100 μg groups, there was a high titre of IgE, but a low titre of IgG2a. In contrast, the 1000 μg group showed the highest IgG2a titre and the lowest IgE titre. *Not detected. †Significant difference at $P < 0{\cdot}01$.

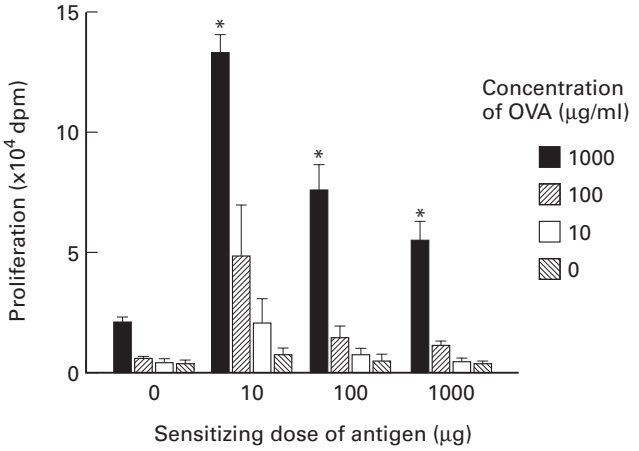

Fig. 4. The proliferative responses of splenic mononuclear cells (SMNC) to stimulation with ovalbumin (OVA). SMNC were cultured with 0, 10, 100, or 1000 μg/ml of OVA for 72 h. SMNC from all groups showed proliferation in response to OVA, although the response was smaller with a higher dose of antigen. Values represent the mean ± s.d. of six mice in each group. *$P < 0{\cdot}005$ when compared with the 0 μg group.

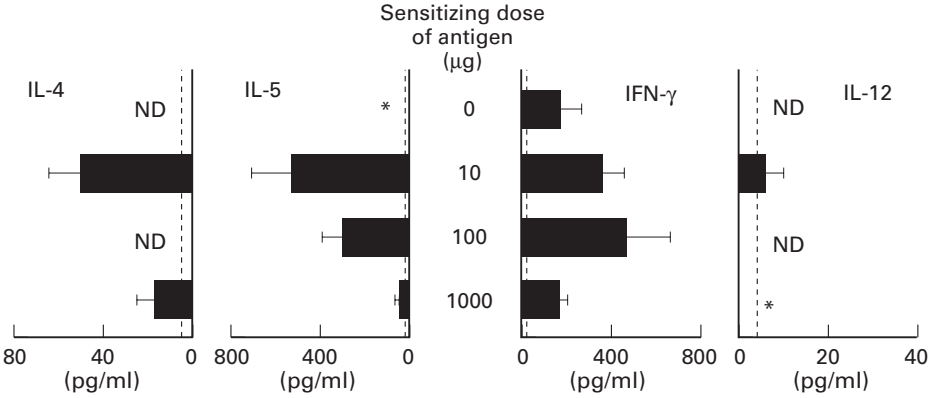

Fig. 5. Production of IL-4, IL-5, IFN-γ, and IL-12 by splenic mononuclear cells (SMNC) cultured in the presence of 1000 μg/ml of ovalbumin (OVA). SMNC from all groups can produced both Th1 (IL-4 and IL-5) and Th2 (IFN-γ) cytokines. IL-12 was barely detectable in all groups. Cytokine production was generally suppressed in the 1000 μg group. However, relative cytokine production was Th2 dominant in the 10 μg group and Th1 dominant in the 1000 μg group. Values represent the mean $\pm$ s.d. of six mice in each group. *Undetectable. ND, Not done.

antigen-induced BHR, and marked production of specific IgE antibody, which are all features of human atopic asthma. We demonstrated that an increased sensitizing dose attenuated all of these features, accompanied by alteration of the cytokine profile in BALF and in the culture supernatant of OVA-stimulated splenocytes. These observations have obvious clinical relevance. Exposure to allergens usually occurs at low concentrations. Our results suggest that such low doses can induce not only an IgE-dominant response but also eosinophilic inflammation, epithelial damage, and development of BHR *in vivo*.

The present results are consistent with previous studies showing that the dose of antigen can influence the development of a specific antibody response, i.e. a low dose of antigen induces the optimum IgE response, whereas a high dose induces an IgG2a response *in vivo* [24]. Production of IgE antibody is dependent on IL-4, a Th2 cytokine [9,10], while IFN-γ released from Th1 cells stimulates IgG2a production, and antagonizes IL-4-induced IgE production [9]. In fact, Th1 cells promote IgG2a production and Th2 cells promote IgE isotype production *in vitro* [35]. However, Arps *et al*. recently reported that the antigen dose-dependent regulation of IgE antibody production is not due to differential polarization between Th1 and Th2 cells [36]. They demonstrated elevated levels of secreted and intracellular IL-4 and IL-10, reduced levels of IL-12, and only slightly elevated IFN-γ levels in cultures of antigen-stimulated spleen cells from CBA/J mice primed with low as well as high doses of antigen (0·1 and 100 μg of keyhole limpet haemocyanin (KLH)). Our results are not markedly different from this report. The amount of IFN-γ produced by SMNC from the 10 μg group was actually larger than in the 1000 μg group, and that produced by SMNC from the 1000 μg group was comparable to the level produced by cells without sensitization (0 μg group). Furthermore, there was no significant inverse correlation between SMNC production of IL-5 and IFN-γ. However, the overall production of Th2 cytokines (IL-4 or IL-5) and Th1 cytokines (IFN-γ) suggested Th2 dominance after low-dose sensitization *versus* Th1 dominance after high-dose sensitization. In fact, the ratio of IL-5 to IFN-γ production by SMNC was significantly correlated with BALF eosinophilia in the present study. It should be noted that these cytokines may be derived from immature Th1- or Th2-like cells rather than mature Th1 or Th2 cells, or may partially be produced by non-$CD4^+$ T cells such as $CD8^+$ $\gamma\delta^+$ T cells, which could be induced by antigen inhalation and then produce IFN-γ [37].

On the other hand, the pattern of cytokines in BALF was more obvious. Both IL-4 and IL-5 (Th2 cytokines) were found in BALF from mice with low-dose but not high-dose sensitization, while IFN-γ was only found in BALF from mice with high-dose sensitization. IL-12 is presumably derived from macrophages, which would explain why it was barely detected in the supernatant of SMNC. IL-12 was detected in BALF after both low- and high-dose sensitization, although the level was higher in the high-dose group. These results may indicate that Th1/Th2 dominance was

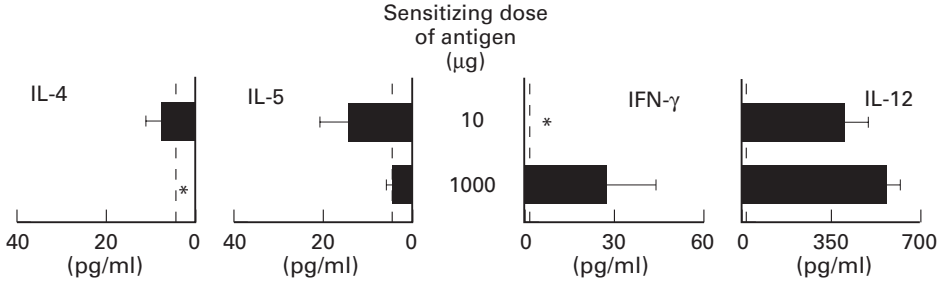

Fig. 6. Levels of IL-4, IL-5, IFN-γ, and IL-12 in bronchoalveolar lavage fluid (BALF) from mice sensitized with 10 or 1000 μg of ovalbumin (OVA). IL-4 and IL-5 were detected in BALF from the 10 μg group but not the 1000 μg group, whereas IFN-γ was detected in the 1000 μg group but not in the 10 μg group. IL-12 was detected in both groups, but the level was higher in the 1000 μg group. Values represent the mean $\pm$ s.d. of six mice in each group. *Undetectable.

more marked in the local response (i.e. in the lungs) than in the systemic response (i.e. in the spleen). In conclusion, the dose of antigen is a major determinant of the polarized development of Th1 and Th2 cells *in vivo*, a result which is consistent with previous *in vitro* studies [25,26].

Like IgE production, we also demonstrated that the extent of eosinophilic inflammation and BHR were associated with the antigen dose. Eosinophilopoiesis induced by IL-5, and expression of both vascular cell adhesion molecule-1 (VCAM-1) and eotaxin by IL-4 would contribute to Th2-induced eosinophilic inflammation [6–8,38,39]. Decreased pulmonary infiltration of eosinophils may be related to attenuation of BHR [40]. It is known that BHR can be induced by adoptive transfer of Th2 but not Th1 clones [41]. Increased levels of IL-12 in the BALF after high-dose sensitization may decrease IL-4 and IL-5 levels as well as increasing IFN-γ production, and thus contribute to inhibition of both airway eosinophilia and BHR [42,43]. In any case, there was no characteristic Th1 response in the 1000 μg group, or rather the Th1 response only contributed to attenuating the Th2 response in our experimental system.

In the airways goblet cell hyperplasia and mucus production were observed in the 10 μg group, but were less marked in the 1000 μg group. Henderson *et al*. demonstrated that leukotriene D_4 could induce mucus secretion and that specific inhibitors of leukotriene production blocked airway mucus release in a mouse model [30]. According to their report, these parts of the allergic response can be initiated before the migration of eosinophils or lymphocytes across the epithelial layer [30]. In addition, the local over-expression of IL-4 within the lungs enhances mucus glycoprotein synthesis by altering gene expression, resulting in the accumulation of mucus glycoprotein in non-ciliated epithelial cells and release of mucus into the airway lumen [44]. Such a cascade of events may be involved in these phenotypic changes, and the sensitizing antigen dose could be one factor modulating the cascade, which may or may not operate through altering the balance of Th1/Th2 cytokines.

It should also be noted that cytokine production by T cells can be modulated via differences in activation induced by different concentrations of antigen or immobilized anti-CD3 MoAb. Allergen-specific $CD4^+$ memory T cells from allergic patients produce high levels of IL-4 when stimulated with a low concentration of allergen, but produce little IL-4 in response to a high concentration of the same antigen [45]. Some established Th2 clones produce IL-2 and IFN-γ upon stimulation with higher but not lower doses of immobilized anti-CD3 MoAb [46]. However, our study found no differences in the pattern of cytokine production on stimulation with different concentrations of OVA *in vitro*. This suggests that the sensitizing antigen dose was an important determinant of the pattern of cytokine secretion in our experimental model.

Although the sensitizing doses used in previous murine studies ranged from 10 to 100 μg [27–32], the present study suggested that sensitization with 10 μg might be more effective than with 100 μg. In conclusion, the dose of the sensitizing antigen was a major determinant of phenotypic changes in a murine asthma model, presumably by affecting the production of Th1 and Th2 cytokines. A higher antigen dose induced a Th1-dominant pattern of cytokine production and attenuated phenotypic changes such as eosinophilic infiltration and epithelial damage in the lungs, antigen-induced BHR, and specific IgE production, with a default increase of IgG2a production.

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